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SECURITY CLASSIFICATION OF THIS PAGE

# REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

AD-A216 904 DTIC ELECTE JAN 16 1990 S D Cs

1b. RESTRICTIVE MARKINGS

3. DISTRIBUTION/AVAILABILITY OF REPORT
unlimited

4. PERFORMING ORGANIZATION REPORT NUMBER(S)

5. MONITORING ORGANIZATION REPORT NUMBER(S)
AFOSR-TR. 89-1687

6a. NAME OF PERFORMING ORGANIZATION
George Washington Univ.

6b. OFFICE SYMBOL (If applicable)

7a. NAME OF MONITORING ORGANIZATION
AFOSR/NL

6c. ADDRESS (City, State, and ZIP Code)
Office of Sponsored Research
2121 "I" Street, NW
Washington DC 20052

7b. ADDRESS (City, State, and ZIP Code)
Building 410
Bolling AFB
Washington DC 20332-6448

8a. NAME OF FUNDING/SPONSORING ORGANIZATION
AFOSR

8b. OFFICE SYMBOL (If applicable)
NL

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
AFOSR-88-0016

8c. ADDRESS (City, State, and ZIP Code)
Building 410
Bolling AFB, DC 20332

10. SOURCE OF FUNDING NUMBERS

| PROGRAM ELEMENT NO. | PROJECT NO. | TASK NO | WORK UNIT ACCESSION NO. |
|---|---|---|---|
| 61102F | 2312 | A5 | |

11. TITLE (Include Security Classification)
Free Radical Mechanisms of Xenobiotic Mammalian Cytotoxicities

12. PERSONAL AUTHOR(S)
Dickens, Benjamin Fisher

| 13a. TYPE OF REPORT | 13b. TIME COVERED | 14. DATE OF REPORT (Year, Month, Day) | 15. PAGE COUNT |
|---|---|---|---|
| ANNUAL | FROM 88/11/01 89/11/30 | '89/11/30 | 8 |

16. SUPPLEMENTARY NOTATION
Keywords: Toxicity, Cytotoxin. (SDW)

| 17. COSATI CODES | | | 18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number) |
|---|---|---|---|
| FIELD | GROUP | SUB-GROUP | Free Radical, IRP volume I and II chemicals, cytotoxicity membrane lipid peroxidation, spin trapping, ESR, cell culture, endothelial cell, smooth muscle cell |
| | | | |
| | | | |

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Our initial goal was to identify if free radical mechanisms are involved in the cytotoxicity of a number of IRP volume I and II chemicals. We found that a number of these agents act to enhance membrane lipid peroxidation in response to a standard dose of exogenous free radicals. Using chlorinated hydrocarbons (carbon tetrachloride, trichloroethylene, dichloroethylene, trichloroethane, dichloroethane) as a model for other IRP chemicals, we established conditions to measure lipid peroxidation in cultured smooth muscle and endothelial cells. These agents induced lipid peroxidation in the presences of physiological levels of iron in these vascular cells by a mechanism that doesn't require cytochrome P-450. Antiradical treatement with deferoxamine and Probucol (but not SOD, catalase, or mannitol) appear to reduce the toxicity of these agents. We have also detected the presences of free radicals in the cultured cells by ESR spin trapping following exposure to iron and chlorinated hydrocarbons. Although this free radical production does not appear to require biotransformation by cytochrome P-450, it is also not a result of spontanous oxidation of the IRP chemicals. Instead, it appears that

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION

22a. NAME OF RESPONSIBLE INDIVIDUAL
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22c. OFFICE SYMBOL
NL

DD Form 1473, JUN 86 Previous editions are obsolete.

90 01 11 133

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

20030205035

# 24 Month Progress Report for AFOSR-88-0016

# "Free Radical Mechanisms of Xenobiotic Mammalian Cytotoxicities"

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Projected Duration
November 1, 1987 to October 31, 1990

Submitted, November 30, 1989

(A) Personal

No changes in personnel have occurred since the last progress report. Dr. Dickens and Dr. Weglicki attended the "Cell Biology of Trauma" workshop sponsored by the Office of Naval Research on September 28, 1989 in Baltimore. At this meeting, we had an opportunity to discuss current research with other DOD researchers. The idea for using a colony formation assay for xenobiotic studies (see discussion below) came about from discussions at this meeting. Both PI's also attended FASEB in New Orleans and the 2nd Annual Oxygen Club Dinner in Bethesda where part of this research was presented.

(B) Facilities

The Bruker NMR mentioned in the 18 month progress report was received in October. It is has been installed and is currently up and running. Research time is being made available for the development of NMR methods which may prove helpful in these studies.

(C) Scientific Progress

SUMMARY:

Two major findings obtained during the second year of this project: the first was that chlorinated hydrocarbons mixed *in vitro* with cumene hydroperoxide - in the complete absences of transition metals - was able to directly activate the hydroperoxide into an alkoxyl radical; the second was the obtaining of direct spin trapping evidence for free radical production within cultured cells following exposure to selected IRP chemicals.

In the first year progress report we proposed the hypothesis that free radical-induced injury participates in the pathogenic mechanism of chlorinated hydrocarbons in mammalian cells. This hypothesis was based upon indirect results obtained from lipid peroxidation since, at that time, we were experiencing a number of technical difficulties with spin trapping studies. However, recent advances in the design of our spin trapping studies have provided strong evidence in support of this hypothesis. This progress report will review the new spin trapping evidence and outline the direction this research project is taking for the third year.

PROPOSED MECHANISM OF CHLORINATED HYDROCARBON TOXICITY:

The possibility that toxic hydrophobic chemicals, such as those of the IRP series, are able to interact directly with macromolecules should not be surprising: many researchers have shown these agents are frequently able to directly bind nucleic acid -- a property associated with their mutagenic properties. However, the interaction proposed between halogenated hydrocarbons and lipid hydroperoxides, and the

resulting acceleration of the "autocatalyzed" (in this case, halocarbon solvent catalyzed) chain of lipid peroxidation was a surprise. The mechanism, originally proposed in the 1st year progress report and modified in the 18-month report, is repeated below.

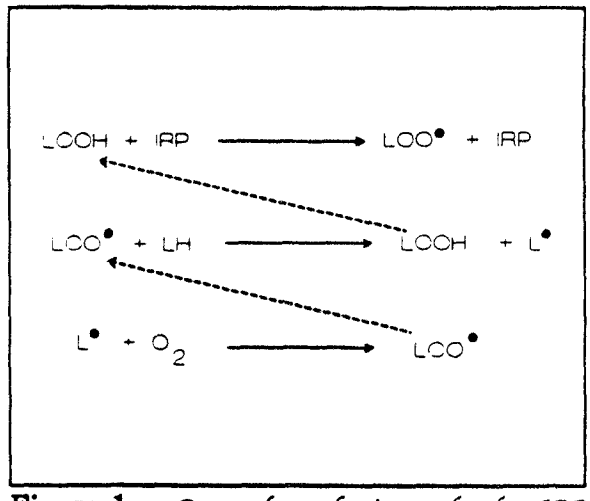


**Figure 1.** *Proposed mechanism whereby IRP chemicals can directly activate, and continue to participate in the "auto-oxidation" of lipid hydroperoxides.*

ESSENTIAL PROGRESS DURING THE SECOND YEAR:

The first major development this year was the development of the *in vitro* assay described in the 18-month progress report to test the hypothesis that chlorinated hydrocarbons, and perhaps other IRP chemicals, are able to directly interact with lipid hydroperoxides to form toxic free radicals. Initially, we thought that the free radicals so formed would be primarily derived from the halocarbons. Our initial studies, summarized in the 18-month report, clearly demonstrated that a alkoxyl (LOO· or perhaps a hydroperoxyl LO·) radical was being rapidly formed from the lipid hydroperoxide following exposure to the halocarbon. The $^{13}C$-experiment described for chloroform in the last report have now been extended to TCE and Carbon Tetrachloride with the same results: the radical adducts are derived not from the IRP chemical but rather the lipid hydroperoxide. These experiments provide a rapid method of screening which IRP chemical are capable of promoting "activation" of lipid hydroperoxides to lipid radicals. For these experiments, the cumene hydroperoxide is dissolved in a solution of the IRP chemical to be tested in the presences of the spin trap, PBN. This non-physiological system provides both advantages and disadvantages: By using organic solvents, we are certain that iron is not playing a role in the production of free production; on the other hand, what happens in total solution of carbon tetrachloride, or acetone, may not be indicative of what occurs in biological membranes. The following IRP

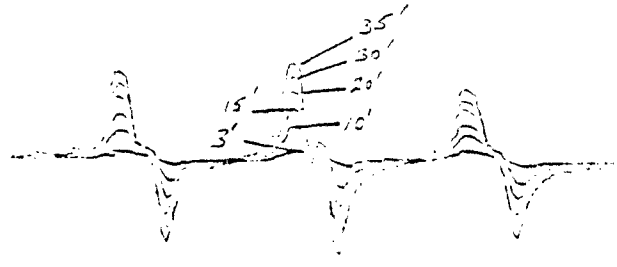


**Figure 2.** *Time course of ESR signal from a mixture of chlorobenzene, PBN, and cumene hydroperoxide.*

compounds have proven to be able to induce free radical production when tested with cumene hydroperoxide by this method. In this table, the difference between "strongly reactive" and "Reactive" is derived from a subjective judgement based upon the length of time necessary to see an ESR signal. Those classified as strongly reactive gave a signal within seconds to a couple of minutes. Those called reactive often provide a very strong ESR signal, but it took 15 to 60 minutes to accumulate. The time course of a slow signal from Chlorobenzene is shown in figure 2. This leads to a second question, what happens to the IRP chemical when it activates the lipid hydroperoxide? The presences of an extractable hydrogen seems to be important, but not absolutely required. This assumption is based upon the observation that carbon tetrachloride and 1,1,2,2 tetrachloroethene took a relatively long time to "activate" the lipid hydroperoxide while chloroform, 1,3-dichloropropane, and TCE did so quite rapidly. It is interesting to note that while benzene was a complete failure in activating the cumene hydroperoxide, chlorobenzene and m-dichlorobenzene were almost in the strongly active class.

# TABLE I

| Strongly Reactive* | Reactive | Not Reactive |
|---|---|---|
| Dichloromethane(+) | m-dichlorobenzene(+) | Acetone |
| Trichloroethane | Chlorobenzene(+) | Hexane |
| Trichloroethylene | Carbon Tetrachloride | Ethylene glycol |
| Dichloroethane | 1,1,2,2-tetrachloroethene(-) | o-chlorophenol |
| Chloroform(+) | n-butyl Phthalate(-) | Xylene |
| Methyl ethyl ketone | | Benzene |
| 1,3-Dichloropropane(+) | | Di-methylphenol |

** In Table I, a (+) following a name means it was more strongly active than the average chemical in that group while a (-) means that is was reactive than chemicals of the same group.*

The second advance using spin trapping within this project was the development of a spin trapping method by which IRP-induced free radicals are trapped following exposure of cultured cells to these chemicals. The initial problem with *in vitro* spin trapping studies is that the spin trap agents, when used in concentrations high enough to trap sufficient free radicals for detection in the ESR spectrometer, are highly toxic. In a recent manuscript involving cultured endothelial cells and the spin trap DMPO from our laboratory following anoxia, 50% of control cells were killed by the spin trap (Arroyo et al, Free Radical Research Communication, 1989). This problem was exacerbated when IRP

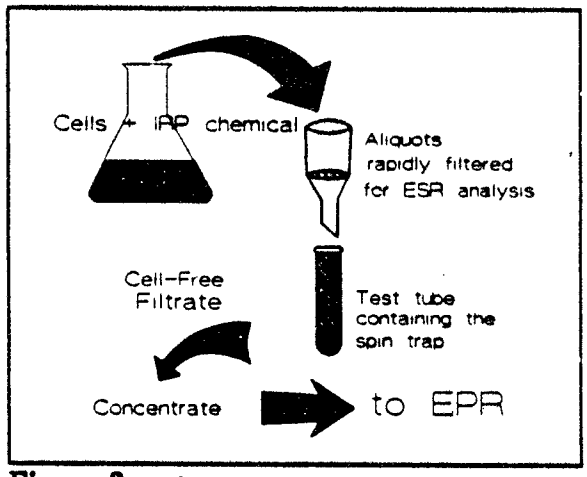


**Figure 3.** *Cells are exposed to IRP chemicals for various times. Cell-free filtrate is collected in a tube pre-loaded with spin trap. The sample is then concentrated into toluene, and analyzed by EPR.*

chemical were added, with virtually 100% of the cells exposed to both spin trap and IRP chemicals dying. Since it didn't seem reasonable to carry out experiments where the toxicity of the sampling condition was as great or greater than the experimental stress being tested, we develop a second method of sampling. This procedure, shown diagrammatically in Figure 3, allows us to test for free radical production without problems associated with direct exposure of cells to spin trapping agents. The cells are exposed to IRP chemicals. Once again a low level of Fe-ADP is included in the incubation. At various time intervals (signals usually found around 40 minutes of incubation), a aliquot is removed and rapidly filtered to remove the cells. The filtrate is collected in a tube that has been pre-loaded with a high concentration of spin trap. Since the spin trap never comes in contact with the cells, we can use as high a concentration as we need. The spin trap is then extracted into toluene, concentrated under dry nitrogen, and then analyzed by ESR techniques. The disadvantage of this method is that very short-lifed radicals, such as the hydroxyl radical, can not be detected by this technique. However, in prelimary evidence from our laboratory with anoxic-reoxygenated endothelial cells suggested that lipid radicals could be detected by this procedure (data in 18-month progress report). By using this method, we were able to screen a large number of IRP chemicals for their ability to induce free radical production in cultured smooth muscle and endothelial cells. Not surprisingly, the agents which gave strong ESR signals in the *in vitro* test with cumene hydroperoxide (ie TCE, and Carbon tetrachloride) also provided positive results in these experiments -- providing strong supporting evidence for the current hypothesis. The two chemicals tested so far that give both negative results with the lipid peroxidation study and the cumene hydroperoxide screening (ethylene glycol and toluene) appear to fail to produce spin adducts. Investigation of other IRP chemicals are currently under way. Typical EPR signals are shown in figure 4: Curve A is cells with Fe-ADP but no IRP

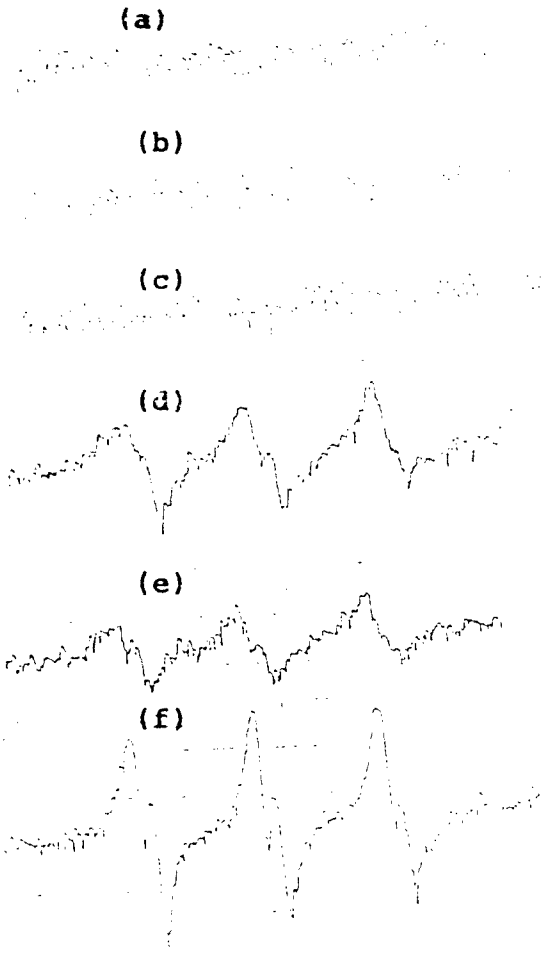


**Figure 4.** *EPR tracings of PBN-adducts obtained from filtrate off of endothelial cells exposed to IRP chemicals.*

chemical, curve B is cells with $CCl_4$ but no iron, curve C is cells plus toluene and Fe-ADP; curve D is cells + Fe-ADP + TCE; curve E is a typical tracing from cells + $CCl_4$ and Fe-ADP; and curve F is the best tracing to date from cells exposed to the conditions from curve E. The following controls were all negative: cell incubations with iron-ADP alone, mixtures of IRP chemicals plus iron-ADP in the absences of incubation with cells, cells incubated without IRP chemicals or iron-ADP. During the course of these studies we have discovered that in the presences of phosphate buffers, PBN-adducts are not stable, while they are stable in bicarbonate buffers. We are therefore making modifications in the experimental protocol which should allow even stronger EPR signals to be obtained from these incubations. It is also worthwhile to point out that while the hyperfine splitting constants varied with the *in vitro* assay described with cumene hydroperoxide depending upon the IRP solvent used (see 18-month progress report), they did not in this biological assay (see curves D-F). The explanation for this seems to be based upon the polarity of the solvent -- in the earlier work the IRP chemical tested was the final solvent the ESR tracing was obtained from. In this experiment, the sample is collected and then extracted into toluene, so that the solvent is always the same. In the case of these toluene extracts, the splitting constants of 13.2 and 2.0 are consistent with a PBN-OL or PBN-OOL adduct. We are trying to use an equation published by Janzen to demonstrate that the radicals detected in both types of experiments are the same.

The protective effect against the toxicity of chlorinated hydrocarbons of two potent antioxidants were tested in our culture system. Both -tocopherol and probucol appeared to partially ameliorate the toxicity of these compounds, but the results were not conclusive. The problem appears to be with the assay we were using to determine toxicity following long-term culture. To date, our assay has just been the ability of exposed cells plated at a 1/4 dilution to grow to confluency. Two problems have cropped up with this naive assay: the first is that no allowance was made for the difference in time it took for one group of cells to reach confluency versus another; second, frequently the cells stop growing all together after one passage and thus never reach a second passage. After discussions with fellow researchers at the ONR sponsored cell trauma workshop, I decided to use a colony forming assay to quantify the toxicity of the various xenobiotics and the ameliorating effect of various antioxidants. In this assay, cells will be initial exposed to each agent ± antioxidant. The effect of the dose of treatment on the number of colonies formed will then be measured. This method should provide us with a measurable endpoint from which to statistically determine if antioxidant treatments can limit the injury caused by IRP chemicals.

## FUTURE STUDIES NOT DISCUSSED IN THE 18-YEAR PROGRESS REPORT

(A) The finding that halogenated hydrocarbons are capable of interacting directly with lipid hydroperoxides raises an interesting question: do halogenated drugs work the same way? Such a mechanism may exist and could explain the cytotoxicity associated with many chlorinated pharmaceutical agents. The *in vitro* test using cumene hydroperoxide will be used to screen a number of other chlorinated agents,

including commonly prescribed drugs, in an effort to determine which structural factors influence this interaction.

(B) In a couple of very preliminary experiments, it appears that cellular glutathione levels are reduced by chronic exposure to carbon tetrachloride and trichloroethylene. These experiments will be extended to see if this trend is significant and to determine which other IRP chemicals may have similar effects.